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Glutathione and Phospholipid Depletion of Liver Tumors After Arterial Ischemia

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Breakdown of membrane phospholipids is a causative event leading to irreversible cell injury after ischemia and reperfusion insults, which might be one mechanism leading to liver tumor cell death after repeated arterial ischemia as well. After 2 hr of hepatic dearterialization followed by 30 min of reperfusion tumor phospholipid was measured chromatographically, glutathione (GSH) analyzed by determining nonprotein sulfhydryl and activity of glutathione-S-transferase (GST) determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as the subtrate. A transient, arterial ischemia for 2 hr induced a substantial decrease of phosphatidylserine (PS) and phosphatidylinosital (PI) compared with sham treatment ($P < 0.01$). Although phosphatidylcholine (PC) and phosphatidylethanolamine (PE) did not significantly decline after a single arterial ischemia for 2 hr, they dropped dramatically following repeated arterial ischemia for 2 hr during 5 days ($P < 0.01$ and $P < 0.05$ respectively). GSH was depleted in tumors after both a single ($P < 0.01$) and repeated arterial ischemia ($P < 0.05$) and GST was inactivated as well ($P < 0.001$). By contrast, neither liver phospholipid nor liver GSH or GST was significantly changed. Tumor growth was significantly retarded in rats subjected to repeated arterial ischemia compared with sham treatment ($P < 0.01$). Repeated arterial ischemia facilitated degradation of tumor membrane phospholipids and induced depletion of GSH and inactivation of GST without affecting the normal liver. Thus, ischemia/reperfusion induced depletion of membrane phospholipids and of GSH might represent two mechanisms by which repeated arterial ischemia led to tumor growth delay.

KEY WORDS: GSH, GST, phospholipid, dearterialization, liver tumor

INTRODUCTION

Hepatic tumor necrosis and regression can be induced by occlusion of its artery supply, since it is mainly nourished by the hepatic artery [1]. Repeated dearterialization as a palliative therapy for unresectable hepatic malignancies seems promising, complete and partial responses to which have been achieved [2]. The specific sequence of events leading to damage and eventual death of tumor cells after repeated arterial ischemia remains to be established. Phospholipid degradation causes irreversible cell injury after a prolonged ischemic insult [3–6] and reintroduction of blood flow leads to additional cellular injury [7]. Reactive oxygen species (ROS) released during reperfusion have been implicated as the mediator responsible for reperfusion injury [8]. GSH, a tripeptide nonprotein thiol amply present in the liver, is an important endogenous antioxidant that has been found to decline after

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ischemia and consequently used as an index of oxidative stress [9]. Ischemia and reperfusion also inactivate enzymes using GSH as substrate against ROS [10]. GST is one of the enzymes but has not been fully explored. We have previously shown that repeated, transient arterial ischemia is able to induce tumor necrosis and retard tumor growth [11]. This procedure repeatedly induces alternating tumor ischemia and reperfusion. Therefore, pathophysiological events induced by ischemia-reperfusion episodes may be one of the mechanisms by which repeated tumor ischemia causes tumor necroses and delays tumor growth. It is further known that GSH may reduce the cytotoxic activity of chemotherapeutic agents [12,13]. The alterations in the level of cellular GSH might have clinical implications in ischemic treatment of hepatic tumors when combined with chemotherapy.

This experiment was designed to study the effects of single and repeated arterial ischemia on the metabolism of tumor and liver phospholipid and to measure levels of GSH and activity of GST in order to illustrate the mechanisms leading to tumor cell death.

MATERIALS AND METHODS

Animals and Tumors

Inbred male Wistar/Furth (W/F) rats weighing 200–280 g were used in this experiment. They were housed three per cage and maintained in artificial light for 12 hr (6 AM to 6 PM) and fed normal laboratory food pellets and water ad libitum. N-Methyl-N-nitrosoguanidine-induced colonic adenocarcinoma was used to produce a tumor model as we previously described [11]. Briefly, under ether anaesthesia the abdomen was opened by midline incision and 0.1 ml of a suspension containing 1×10^6 viable tumor cells injected into the left lobe of the liver subcapsularly. Five days after inoculation the rat abdomen was reopened and the longest (1) and the shortest diameter, (2) of the tumors were recorded. Tumor volume (V) was calculated according to the equation: [11]

$$V = a \times b^2/2.$$

Animals were then randomly allocated to different groups. All animals received humane care according to National Institutes of Health (NIH) Publication 85-23 (revised 1985). This experiment was approved by the Ethics Committee at the Lund University.

Surgical Procedure

Devascularization was carried out sparing only the hepatic artery, the portal vein and the common bile duct and a mini-occluder was simultaneously implanted around the hepatic artery [11]. Repeated arterial ischemia for 2 hr was achieved by inflating the balloon inside the mini-occluder with 0.06–0.08 ml of saline. The surgical procedure of sham dearterialization was the same as above except that the balloon was not inflated.

Rats were sacrificed 30 min after reperfusion in single dearterialization groups and after the last reperfusion in repeated dearterialization groups. The tumor and part of liver tissue were harvested and frozen in liquid nitrogen until analysis. Tumor sizes before and after treatment were recorded for the calculation of tumor growth rates which was expressed as the tumor volume ratio V_5/V_0 (V_5: tumor volume at day 5 and V_0: tumor volume at day 0). Blood samples were obtained just before sacrifice for estimation of aspartate-aminotransferase (ASAT) and alanine-aminotransferase (ALAT).

Assay of Phospholipid

Liver and tumor lipids were extracted with chloroform–methanol (1:1, 20 ml/g tissue) containing 0.005% butylated hydroxytoluene as an antioxidant [14]. After removal of the protein precipitate and washing of the extract, the phospholipids were separated by thin-layer chromatography (TLC) on silica gel G plates developed in chloroform–methanol–acetic acid (100:80:12). The lipids were visualized in an I_2 vapor for a few minutes. By the above steps, the phospholipid subclasses were clearly separated from each other and then scraped into different glass tubes for determining the phosphorus as described [15]. The absorbance of the stable blue color of the samples was read against a reagent blank at 830 nm in 1-cm cells in an ultraviolet (UV)-visible spectrophotometer (Spectrophotometer UV-260, Shimadzu, Kyoto, Japan). For a calibration, 1-μg, 2-μg, and 4-μg phosphorus standards were carried out simultaneously through the procedure. The sample P was then calculated according to the following equation:

$$\mu gP \text{ in sample aliquot} = \text{absorbance of sample} \times \mu gP \text{ in standard/absorbance of standard.}$$

Measurement of GSH

GSH was measured by determining nonprotein sulfhydryl as described [16]. A 50-μl aliquot of the acid abstract, 50 μl of 1 N NaOH and 50 μl of distilled water were mixed with 750 μl of 0.15 M sodium phosphate buffer (pH 7.5). Then 100 μl of 6 mM 5,5′-dithiobis (2-nitro benzoic acid) (DTNB) was added. The absorbance was immediately measured at 412 nm.

GST Assay

GST activity using CDNB as the substrate was quantified spectrophotometrically at 30°C according to Habig et al. [17].

TABLE I. Total and Subpopulations of Phospholipids (μg/mg Fresh Tissue) Following a Single Induction of Arterial Ischemia of the Liver for 2 hr (Mean ± SEM)

	Total PL	PC	PE	PI	PS	CL	PA	LPC
DL (n = 4)	11.82 ± 0.67	6.67 ± 0.46	2.94 ± 0.14	0.67 ± 0.12	0.47 ± 0.02	0.06 ± 0.00	0.48 ± 0.05	0.54 ± 0.11
DT (n = 4)	6.36 ± 0.52*	3.04 ± 0.25	1.78 ± 0.21	0.36 ± 0.06*	0.29 ± 0.02*	0.21 ± 0.09	0.40 ± 0.11	0.37 ± 0.04
SL (n = 3)	9.65 ± 0.83	4.72 ± 0.26	2.39 ± 0.24	0.78 ± 0.10	0.73 ± 0.20	0.13 ± 0.01	0.48 ± 0.02	0.45 ± 0.09
ST (n = 3)	10.42 ± 1.23	3.71 ± 0.57	2.3 ± 0.06	0.65 ± 0.12	0.71 ± 0.07	0.90 ± 0.49	0.79 ± 0.10	0.82 ± 0.19

DL, dearterialized liver; DT, dearterialized tumor; SL, sham-dearterialized liver; ST, sham-dearterialized tumor; Total PL, total phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; PA, phosphatidic acid; LPC, lyso-phosphatidylcholine.
*$P < 0.01$.

TABLE II. Total and Subpopulations of Phospholipids (μg/mg Fresh Weight) After 2 hr of Repeated Arterial Ischemia of the Liver for 5 Days (Mean ± SEM)

	Total PL	PC	PE	PI	PS	CL	PA	LPC
DL (n = 5)	12.14 ± 1.36	6.40 ± 0.88	3.22 ± 0.28	0.49 ± 0.06	0.27 ± 0.08	0.43 ± 0.05	0.95 ± 0.10	0.61 ± 0.04
DT (n = 5)	4.15 ± 0.97	1.35 ± 0.35**	0.75 ± 0.29*	0.39 ± 0.23	0.19 ± 0.09	0.26 ± 0.12	0.30 ± 0.08	0.50 ± 0.10
SL (n = 5)	11.26 ± 0.60	6.05 ± 0.08	2.89 ± 0.19	1.13 ± 0.69	0.15 ± 0.01	0.36 ± 0.04	0.89 ± 0.07	0.62 ± 0.08
ST (n = 5)	6.118 ± 0.67	3.29 ± 0.49	1.21 ± 0.24	0.30 ± 0.03	0.09 ± 0.03	0.26 ± 0.05	0.48 ± 0.04	0.65 ± 0.07

All abbreviations as in Table I.
**$P < 0.01$ and *$P < 0.05$.

Statistical Analysis

Student's *t*-test was used for the evaluation of the phospholipids and aminotransferases and Mann-Whitney U-test for the statistic evaluation of tumor growth delay. Statistical significance was considered if $P < 0.05$.

RESULTS

Phospholipids in tumor and liver tissue after one arterial ischemia for 2 hr are shown in Table I. Total phospholipids in dearterialized tumors were significantly depleted ($P < 0.01$). PC and PE declined but were not significantly less than the control counterparts ($P > 0.05$); PI and PS were the only fractions that decreased significantly ($P < 0.01$ vs control). Total liver phospholipids did not change ($P > 0.05$), nor did the individual fractions ($P > 0.05$).

Table II shows various fractions of phospholipids following repeated arterial ischemia for 2 hr during 5 days. PC and PE were now significantly diminished compared with sham repeat-dearterializations ($P < 0.01$ and $P < 0.05$, respectively). By contrast, neither total liver phospholipid nor its subpopulations was diminished ($P > 0.05$).

GSH, an endogenous antioxidant, is a good index for the release of oxygen-derived free radicals. It was significantly depleted after 30 min of reperfusion following 2 hr of arterial ischemia performed either once or repeatedly ($P < 0.01$ and $P < 0.05$, respectively) in tumors indicating that free radicals were released (Table III). The activity of GST was also diminished ($P < 0.001$). However, liver GSH and GST were not affected by arterial ischemia ($P > 0.05$).

Repeated arterial ischemia performed repeatedly for 2 hr during 5 days significantly delayed tumor growth when compared with sham treatment, as shown in Figure 1 ($P < 0.01$) and coincided with a loss of structural PC and PE and decrease of GSH and GST.

ASAT and ALAT are shown in Figure 2. There was no significant difference between treatments and controls ($P > 0.05$), which was also in agreement with unchanged phospholipids, GSH and GST, in liver tissues.

DISCUSSION

The present study showed that arterial ischemia facilitated phospholipid degradation in liver tumors. Total phospholipid in dearterialized tumors was selectively depleted. PI and PS were more significantly reduced than the other subgroups of phospholipids following a single dearterialization. Repeated dearterializations for 2 hr during 5 days induced significant depletion of PC and PE accompanied by delayed tumor growth. Moreover, tumor GSH was diminished and GST inactivated following transient dearterialization performed either once or repeatedly.

Phospholipids are major components of cell membrane bilayer, degradation of which characterizes irreversible

TABLE III. GSH Levels and GST Activities After Single Episode of Arterial Ischemia and After Repeated Arterial Ischemia (Mean ± SEM)

	GSH (nmol/mg protein)		GST (μmol/min/mg protein)	
	Tumor	Liver	Tumor	Liver
A single ischemia				
Control (n = 6)	15.86 ± 4.56	32.10 ± 7.78	0.72 ± 0.06	1.13 ± 0.08
Treatment (n = 5)	2.19 ± 0.97**	19.34 ± 4.19	0.34 ± 0.04***	0.95 ± 0.67
Repeated ischemia				
Control (n = 5)	18.40 ± 5.30	32.17 ± 6.66	0.83 ± 0.06	0.81 ± 0.05
Treatment (n = 5)	5.91 ± 2.59*	38.96 ± 3.26	0.30 ± 0.07***	0.67 ± 0.03

GSH, glutathione; GST, glutathione-S-transferase. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with corresponding controls.

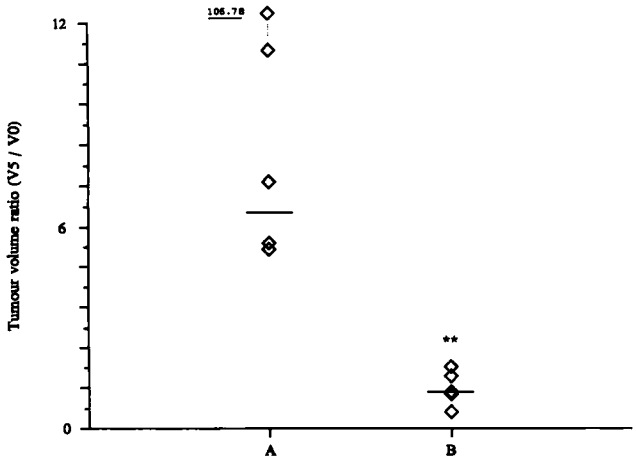

Fig. 1. Tumor volume ratio before and after repeated dearterializations for 5 days. Sham dearterialization (**A**); repeated dearterializations (**B**). $**P < 0.01$ compared with A.

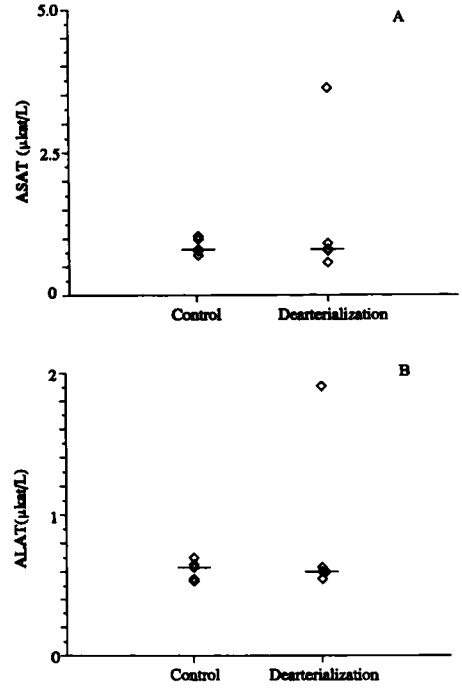

Fig. 2. **A:** Aspartate-aminotransferase (ASAT). **B:** Alanine-aminotransferase (ALAT) following repeated dearterializations for 5 days (mean ± SEM). No significant releases of ASAT and ALAT could be demonstrated after dearterializations compared with the controls ($P > 0.05$).

injury following tissue ischemia [3–6]. However, break down of phospholipid depends on the period of ischemia. Less than 1 hr of ischemia induces only slight loss of phospholipid with a recoverable mitochondrial depression and without change of Ca^{2+} permeability [3,18]. Two or 3 hr of ischemia produces a 40–50% phospholipid decrease with considerable membrane dysfunction, such as 25- to 50-fold increase in membrane permeability to Ca^{2+} [2]. Phospholipase A_2 (PLA_2) has been suggested to be the principal phospholipid degradation enzyme based on the observation that PLA_2 inhibitors reduce ischemic injury [3,6,19]. More recently, PI-specific phospholipase C (PL-C) has also been suggested to be involved in the speedy degradation of phospholipid because an increased PI breakdown can be discovered during reperfusion of ischemic brain and myocardium [20,21]. An imbalance between deacylation and reacylation of membrane phospholipids and inhibition of de novo synthesis are other factors that may affect net phospholipid loss following ischemia [22]. The phospholipid breakdown not only increases membrane permeability but also destroys a number of enzymes responsible for the maintenance of cellular homeostasis in the membrane phospholipid bilayer [22]. Thus, it is conceivable that phospholipid depletion leading to membrane dysfunctions plays a key role in ischemic injury. We have previously found that the optimal period of repeated arterial ischemia is between 2 and 3 hr when performed daily [11]. Repeated arterial ischemia for 1 hr is not as efficient as 2 or 3 hr to arrest tumor growth [11]. Repeated arterial ischemia for 30 min does not alter tumor growth at all [11]. This agrees with the observation that ischemia for 30 min does not noticeably change phospholipid composition [3,22] but following 2 or 3 hr of ischemia a 40–50% loss of phospholipids is seen [3].

Two hours of dearterialization repeated for 5 days elicits a considerable loss of total phospholipid, especially a reduction of PC and PE concomitant with a significant tumor growth delay as demonstrated in this study. These findings indicate that an irreversible cell injury has been produced. A single arterial ischemia, however, failed to produce PC and PE depletion, which might suggest that a single arterial ischemia did not irreversibly destroy tumor cells. Our result implies that dearterialization has to be performed for 2 or 3 hr and has to be repeated daily in order to be effective.

Arterial ischemia delivered once or repeatedly for 5 days did not alter liver phospholipid and correlated with normal aminotransferases as well. The liver is unique in having a double blood contribution; the portal vein providing about 70–75% and the hepatic artery 25–30% of the total blood supply with each vessel supporting about 50% of the oxygen needed in the liver. The liver tissue is very sensitive to oxygen depletion. Permanent dearterialization that reduces liver oxygen supply by one-half is often associated with more complications [1]. Transient dearterialization reduces liver blood flow as well, but only for a short period [23]. Furthermore, extraction of oxygen from the portal blood might compensate for the oxygen drop during a temporary dearterialization [24]. Depletion of tumor but not liver phospholipids indicated that repeated and transient arterial ischemia selectively produced tumor ischemia while sparing liver parenchyma. Repeated arterial ischemia therefore might have a less injurious effect on normal liver tissues, as we have previously demonstrated [11].

During the latest decade, it has been found that part of ischemic damage reveals at reoxygenation. This damage is referred as reperfusion injury and ROS are the mediators responsible for it [25,26]. On reintroduction of oxygen, the hypoxanthine accumulated during ischemia is oxidized by xanthine oxidase to produce an excessive amount of superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) [27], which then is converted to the most reactive hydroxyl radical, OH· [28]. GSH, a substrate of glutathione peroxidase [29], cannot only break the reaction leading from the superoxide radical to the highly reactive hydroxyl radical but also react directly with the superoxide radical to produce the relatively inert glutathione radical [30]. GSH is found to decline during ischemia [31] and further decrease following 60 min of reperfusion in rat liver due to its scavenging of ROS [32]. Our observation that GSH in tumor tissue declined significantly after arterial ischemia for 2 hr, followed by 30 min of reperfusion, indicated a release of ROS. Thus, reperfusion injury was also induced in liver tumors following arterial ischemia. Ischemia and reperfusion compromise that antioxidative defense system by inhibiting activities of enzymes that use GSH as substrate to reduce oxygen free radicals as well [10,33]. In our experiment, inactivation of GST is consistent with these findings and implies that the antioxidant defense system in tumors is impaired. GSH is necessary for cell proliferation, and depletion of tumor GSH by L-buthionine (SR) sulfoximine (BSO) has been shown to retard tumor growth [34], which might be another mechanism responsible for tumor growth delay after ischemic therapy. GSH and GST are also known to participate in the detoxification of electrophilic xenobiotics. A correlation appears to exist between an elevated cellular level of GSH and activity of GST and resistance to alkylating agents [35]. This is thought to occur because alkylating agents are inactivated by a reaction with GSH [36] catalyzed by GST [37]. Consumption of GSH and inactivation of GST following hepatic ischemia would hypothetically increase tumor susceptibility to alkylating agents.

CONCLUSION

Repeated dearterialization elicited tumor phospholipid decline that correlated with tumor growth delay in comparison with sham treatment. Our results may help to better understand the mechanisms leading to tumor necrosis and growth delay following repeated arterial ischemia and have implications for clinical practice of ischemic therapy, especially in its combination with cytotoxic agents. Moreover, ischemia-reperfusion injury might be augmented by pretreatment with BSO by which GSH is further reduced.

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COMMENTARY

M. Satya Murthy, PhD†: Perhaps the authors could let us know how soon after ischemia a change in tumor size is noticeable.

Dr. Wang: We have no idea how long after ischemia a change in tumor size can be observed. Nevertheless, we do know that tumor regression cannot be induced by a single occasion of ischemia. Repeated ischemia is needed to induce a change in tumor size. Tumor size changed after the last ischemia, even checked immediately after the last treatment, because it is the result of serial inductions of ischemia and not of the last induction.

Dr. Murthy: Please explain why, in Figure 2A,B, the values in the dearterialization group are higher than in the controls.

Dr. Wang: High release of ASAT and ALAT was only found in one rat subjected to repeated dearterialization. The rest were all normal and the medians of those two groups are almost at the same level. As far as statistical significance is concerned, no difference exists between the two groups.

Dr. Murthy: How do you define irreversible damage? How does reperfusion injury add to the already irreversible damage?

Dr. Wang: Irreversible damage induced by ischemia means that cells are so severely injured that it will not recover, even if blood flow is restored, but continue to degenerate and eventually become necrotic. Reperfusion injury is recommended since it is found that much of the ischemic injury occur not during ischemia but during reperfusion. However, reperfusion injury is not an isolated event independent of ischemia but is dependent on the period of ischemia. Reactive oxygen species (ROS) is believed to be the initiator of reperfusion injury. Xanthine oxidase (XO) is an important source of superoxide in reperfused tissue. Under normal conditions, xanthine-utilizing enzymes exist predominantly as xanthine dehydrogenase (XD), which use NAD^+, rather than oxygen, as an electron acceptor and produce NADH rather than superoxide. During prolonged ischemia, proteolytic conversion of XD to XO might be triggered by an elevation of cytosolic Ca^{2+} concomitant with hypoxanthine accumulation resulting from ATP degradation. On reintroduction of oxygen, the hypoxanthine is oxidized by XO producing an excessive amount of superoxide anion and hydrogen peroxide. A severe hepatic ischemia is a prerequisite for a significant intracellular formation of ROS during reperfusion. Less than $1\frac{1}{2}$ hr of ischemia does not induce any conversion of XD to XO in the rat liver. Thus, reperfusion injury is clearly a damage originating from prolonged ischemia and revealed during reoxygenation. The irreversible injury here is induced by ischemia and part of its shows as reperfusion injury.

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